ORIGINAL ARTICLE

Antibodies Against Hypocretin Receptor 2 Are Rare in Narcolepsy

Maria Pia Giannoccaro, MD[1,2]; Patrick Waters, PhD[1]; Fabio Pizza, PhD[2,3]; Rocco Liguori, MD[2,3]; Giuseppe Plazzi, MD[2,3]; Angela Vincent, FRCPath[1]

[1]Nuffield Department of Clinical Neurosciences, Oxford University, Oxford, United Kingdom; [2]Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy; [3]IRCCS Institute of the Neurological Sciences of Bologna, Bologna, Italy

Study Objectives: Recently, antibodies to the hypocretin receptor 2 (HCRTR2-Abs) were reported in a high proportion of narcolepsy patients who developed the disease following Pandemrix® vaccination. We tested a group of narcolepsy patients for the HCRTR2-Abs using a newly established cell-based assay.

Methods: Sera from 50 narcolepsy type 1 (NT1) and 11 narcolepsy type 2 (NT2) patients, 22 patients with other sleep disorders, 15 healthy controls, and 93 disease controls were studied. Cerebrospinal fluid (CSFs) from three narcoleptic patients were subsequently included. Human embryonic kidney cells were transiently transfected with human HCRTR2, incubated with patients' sera for 1 hr at 1:20 dilution and then fixed. Binding of antibodies was detected by fluorescently labeled secondary antibodies to human immunoglobulin G (IgG) and the different IgG subclasses. A nonlinear visual scoring system was used from 0 to 4; samples scoring ≥1 were considered positive.

Results: Only 3 (5%) of 61 patients showed a score ≥1, one with IgG1- and two with IgG3-antibodies, but titers were low (1:40–1:100). CSFs from these patients were negative. The three positive patients included one NT1 case with associated psychotic features, one NT2 patient, and an NT1 patient with normal hypocretin CSF levels.

Conclusions: Low levels of IgG1 or IgG3 antibodies against HCRTR2 were found in 3 of 61 patients with narcolepsy, although only 1 presented with full-blown NT1. HCRTR2-Abs are not common in narcolepsy unrelated to vaccination.

Keywords: narcolepsy, antibodies, HCRTR2, autoimmune.

Statement of Significance

Antibodies against HCRTR2 are rare in patients with nonvaccine-associated narcolepsy, are only low titer, and may be more often associated with atypical clinical features. Their pathogenic significance is unclear and needs further investigation.

INTRODUCTION

Narcolepsy is a rare central disorder of hypersomnolence, mainly characterized by excessive daytime sleepiness (EDS) and cataplexy, which is a sudden loss of muscle tone triggered by strong emotions. Other features include hallucinations at sleep onset and awakening and sleep paralysis.[1] Two subtypes of the disease are recognized, narcolepsy type 1 (NT1) and narcolepsy type 2 (NT2). NT1 is associated with loss of hypocretin (HCRT)-secreting neurons in the lateral hypothalamus[2–4] without a parallel loss of neurons expressing melanin-concentrating hormone[3,4] The strong HLA DRB1*06:02 association[5,6] suggests an autoimmune-driven process.[7] Both NT2 and NT1 are characterized by EDS and sleep-onset rapid eye movement periods. However, in NT2 cases, the hypocretin 1 cerebrospinal fluid (CSF) levels should be above the cutoff value of 110 pg/mL or >1/3 of the mean control values,[8] and the HLA DRB1*06:02 association is found in only 40%–50%[9,10] of patients. Therefore, the pathophysiology of NT2 remains elusive.

The role of autoimmunity has been strengthened by the increased rates of narcolepsy onset in children following *Streptococcus pyogenes*[11] and influenza A H1N1 infections[12] as well as after exposure to selected H1N1 vaccine preparations such as Pandemrix®.[13] These observations suggest that, in an appropriate genetic setting, exposure to some infections can lead, through unknown mechanisms, to a hypocretin neuron-specific autoimmune destruction. There is no evidence for disease-specific antibodies directed against neuronal[14–19] or nonneuronal autoantigens[20] in any form of narcolepsy, but a recent study noted homology between the H1N1 influenza virus nucleoprotein A and the first extracellular domain of the hypocretin receptor 2 (HCRTR2). Antibodies to the HCRTR2 (HCRTR2-Abs) were found in 85.0% (17 of 20) of NT1 patients with a history of Pandemrix vaccination, although also in up to 34.7% (16 of 46) of the non-narcolepsy control groups.[21] The diagnostic and pathological significance of the antibodies in vaccine-related narcolepsy is, therefore, not clear.[22] Moreover, other narcolepsy patient groups were not investigated.

We used a live cell-based assay (CBA) approach, as now used in many diagnostic antibody tests,[23,24] to establish an assay for HCRTR2-Abs and tested sera from patients with narcolepsy, other sleep disorders, healthy, and disease controls.

MATERIALS AND METHODS

Patients and Serum Samples

Sera from 61 narcolepsy patients (41 adults and 20 children), including 50 patients with NT1 and 11 patients with NT2, 22 patients with other sleep disorders (11 patients with Idiopathic Hypersomnia [IH] and 11 patients complaining of EDS with normal sleep studies [subjective-EDS, sEDS]), 15 healthy controls (HC), and 93 disease controls (39 epileptic and 54 patients with known positivity for neuronal surface antigens, i.e., AQ4, CASPR2, LGI1, and MOG) were studied. Sleep disorders and epileptic patients' sera and CSFs ($n = 3$) were retrieved from the Sleep Center of the Neurologic clinic of Bologna. Antibody-positive and healthy control sera, all anonymized, were retrieved from the Neuroimmunology laboratory of the Nuffield Department of Clinical Neurosciences. Narcolepsy and IH were diagnosed accordingly to the *International Classification of Sleep Disorders*, 3rd edition.[8] Subjective daytime sleepiness was assessed using the Epworth Sleepiness Scale. All patients underwent Multiple Sleep Latency Test (MSLT) the day after

48 hr continuous polysomnography. CSF HCRT-1 levels and HLA status at locus DQB1*0602 were available in all narcoleptic and other sleep disorder patients. Local ethical committee approved the study, and written informed consent was obtained from all study participants.

Plasmid Construct and Transfection of Human Embryonic Kidney Cells

Human *HCRTR2* complementary DNA in the pcDNA3.1(−) mammalian expression vector was used for transient transfection of human embryonic kidney (HEK) 293 cells. Briefly, HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma®) supplemented with 10% fetal calf serum (Biowest®) and 1% each of penicillin G and streptomycin in 175-cm^3 tissue culture flasks at 37°C, 5% CO_2; 4.6×10^5 HEK cells were seeded into 6-well plates containing four borosilicate glass coverslips (13 mm, VWR International®) previously coated with poly-L-lysine. Twenty-four hours after seeding, at about 60% of confluence, the cells were transiently transfected with the HCRTR2-encoding plasmid. A total of 3 μg of DNA was diluted in 50 μL DMEM, 0.82 μL of 20% glucose, and 1.5 μL of polyethylenimine per well. After 12–16 hr of incubation (37°C), the media was replaced and cells cultured for a further 24 hr before use.

HCRTR2-IgG CBA

A CBA for the HCRT2-Abs was performed as for other routine antibody assays in the Oxford diagnostic laboratory. Briefly, coverslips were incubated either with commercial goat anti-HCRTR2 antibody (ab65093, 1:300 dilution; Abcam) or with patient or control serum (1:20) or CSF (1:3), in incubation buffer (1% bovine serum albumin and 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in DMEM) at room temperature for 1 hr. After washing, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature, washed, and incubated with the appropriate secondary antibodies: donkey antigoat IgG (H+L) Alexa Fluor® 568-conjugated antibody (1:750) or goat antihuman immunoglobulin G (IgG; (H+L)-Alexa Fluor® 568-conjugated antibody (1:750; ThermoFisher scientific).

Table 1—Demographics, CSF Hcrt-1 Levels, and HLA DQB1*0602 Status.

Diagnosis	No. of patients	Sex (female /male)	Age at EDS onset, years, mean ± *SD*	Time to cataplexy, years, mean ± *SD*	Time to sampling, years, mean ± *SD*	HLA-DQB1*0602	Hcrt-1 levels	Additional features; HCRTR2-Ab
NT1	47	17/30	18 ± 11.3	2.6 ± 4.5	12.6 ± 11.1	+	<110	Psychosis (*n* = 3); postvaccine (*n* = 1)
[a]	1	M	15	12	23	+	<110	Psychosis; HCRTR2-Ab positive (IgG3) (Patient 1)
	1	F	14	Simultaneous with EDS onset	1	-	<110	Psychosis
[a]	1	F	16	1	7	-	Normal	HCRTR2-Ab positive (IgG1 > IgG2) (Patient 2)
NT2	5	3/2	27.5 ± 14.1	-	7.7 ± 5.7	-	Normal	
[a]	1	F	Childhood	-	-	-	Normal	HCRTR2-Ab positive (IgG3) (Patient 3)
	4	2/2	12 ± 4.3	-	8 ± 8.1	+	Normal	
	1	M	6	-	18	+	<110	
IH	10	3/7	21.1 ± 16.4	-	20.2 ± 17.3	-	Normal	
	1	F	20	-	11	+	Normal	
sEDS	9	4/5	20.5 ± 13.7	-	10.4 ± 8.1	-	Normal	
	2	1/1	27.5 ± 10.6	-	8.5 ± 9.1	+	Normal	
HC	15	-	-	-	-	NA	NA	
DS	39	-	-	-	-	NA	NA	
AI	54	-	-	-	-	NA	NA	

Abbreviations: Hcrt-1, hypocretin 1; Normal levels >200 pg/ml; NA, not available; DS, disease controls; AI, autoimmune controls.*
[a] Indicates patients positive for hypocretin receptor 2 (HCRTR2)-Abs.

To confirm the presence of IgG antibodies, goat anti-human IgG-Fc (1:750) or mouse anti-human IgG1, IgG2, IgG3, or IgG4 (Sigma; 1:50) were used followed by donkey anti-goat IgG-Alexa Fluor® 568 goat or anti-mouse IgG-Alexa Fluor® 568 conjugated secondary antibody (1:750). The presence of immunoglobulin M (IgM) antibodies was assessed by goat anti-human IgM Alexa Fluor® 568-conjugated antibody (Thermo Scientific, #31125; 1:750).

Immunofluorescence Analysis

Coverslips were coded, and the immunofluorescence was scored for the frequency and intensity of surface antibody binding, using a visual nonlinear scoring system from 0 to 4, as described previously.[25] All samples were tested twice, and any serum showing a score ≥ 0.5 was repeated at least 3 times. Samples scoring ≥1 were considered positive and evaluated for specificity by testing against at least one other antigen.

Statistical Analysis

Frequencies are expressed as percentages. Mean, *SD*, and *SE* were calculated and data were plotted using GraphPad PRISM 6.

RESULTS

Clinical Data

The clinical data, the HCRT-1 levels, and DQB1*0602 status are given in the Table 1. Among narcolepsy patients, 18 (7 children) were considered to have had an acute onset (≤3 months elapsed between EDS and cataplexy onset) and in all but 1 cataplexy and EDS appeared in the same month. The interval between onset and serum sampling, however, was highly variable (see Table 1), and only four acute patients were sampled at 2 (3 patients) and 4 (1 patient) months after onset.

The narcolepsy cohort included rare cases with uncommon combinations of clinical features, HLA status and CSF HCRT-1 levels, as well as atypical cases, including patients with NT1

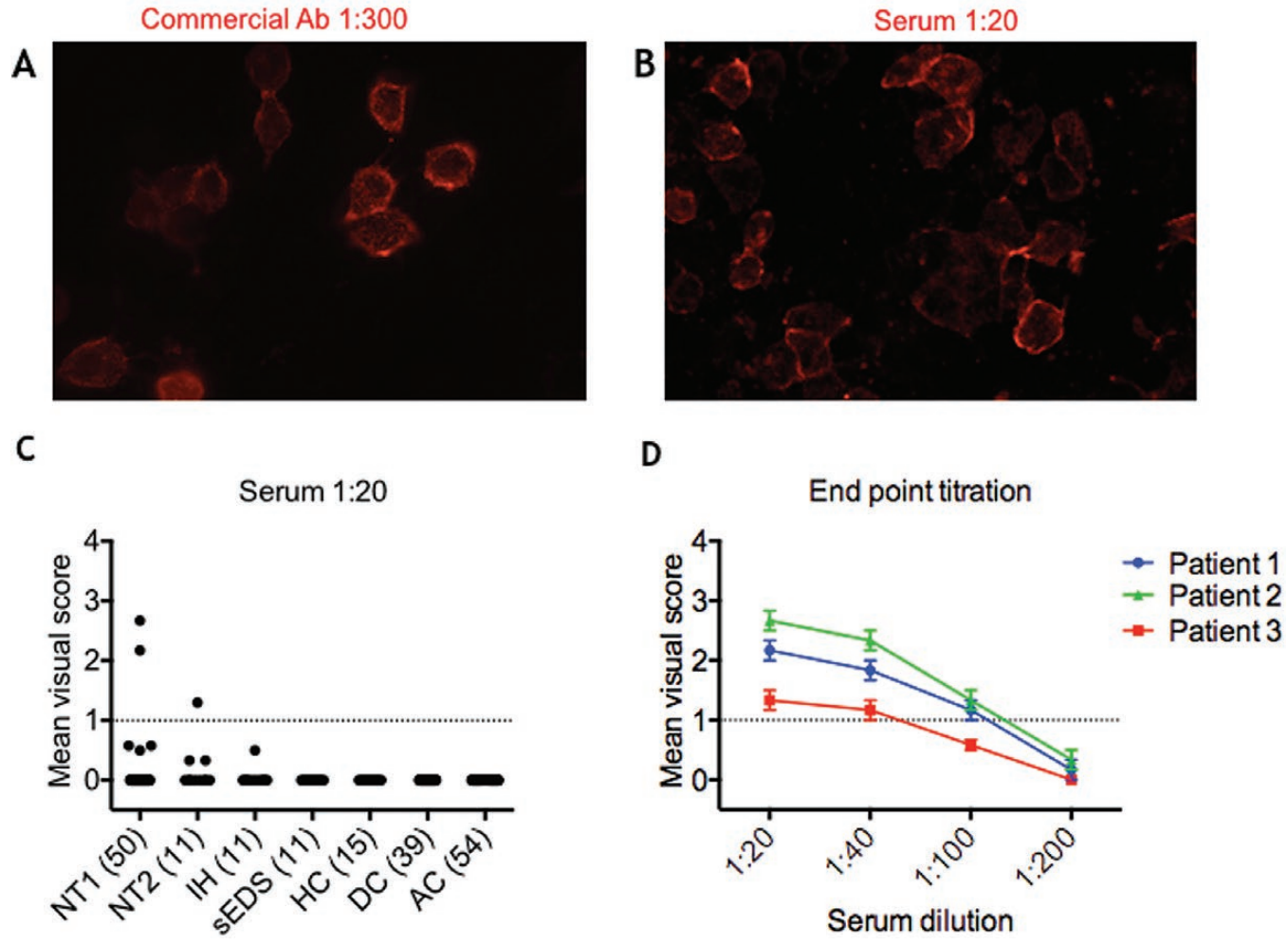

Figure 1—(A) Representative image of a cell-based assay (CBA) using the commercial antibody or (B) a positive patient serum (Patient #1). Binding of antibodies is scored on a scale from 0 to 4 with values 1 and above considered positive.[24] Note that the intensity of binding is similar between A and B, but the commercial antibody has been diluted 300-fold and the patient serum only 20-fold. (C) Screening of the 191 serum samples detected only 3 narcoleptic patients with scores of 1 or above. (D). Serial dilutions of the sera showed that the end point dilutions, giving a score of 1, were only 1:40 or 1:100 indicating low antibody titers. The results are given as mean ± standard error of mean of 3 or more determinations. (D). CBA indicates cell-based assay; DC, disease controls; AI, autoimmune controls.

and associated psychosis (Table 1). One patient presented NT1 after receiving the Focetria® H1N1 vaccine.

HCRTR2 Antibody Detection

HCRTR2 was expressed on the surface of the live HEK cells as shown by strong binding of the commercial antibody against the extracellular domain of the receptor (Figure 1A). All control sera were negative for binding to HCRTR2. Only 3 (5%) of 61 narcolepsy sera showed a positive score of ≥1 (Figure 1B, C). Serum dilutions were performed in the three sera scoring ≥1, and none of them reached a score of 1 at a dilution beyond 1:100 (Figure 1 D). CSFs from the three positive patients were negative.

The presence of IgG antibodies was confirmed using an IgG-Fc secondary antibody and also by determining the IgG subclasses in the three positive patients (Figure 2A, B). IgG3 HCRTR2-Abs were present in two patients (one patient with NT1 and one patient with NT2). The third patient (NT1) had IgG1 and IgG2 HCRTR2-Abs. IgM antibodies were negative in each case (data not shown).

The clinical features of these three patients are summarized in Table 1. Patient #1 presented with a progressive psychiatric

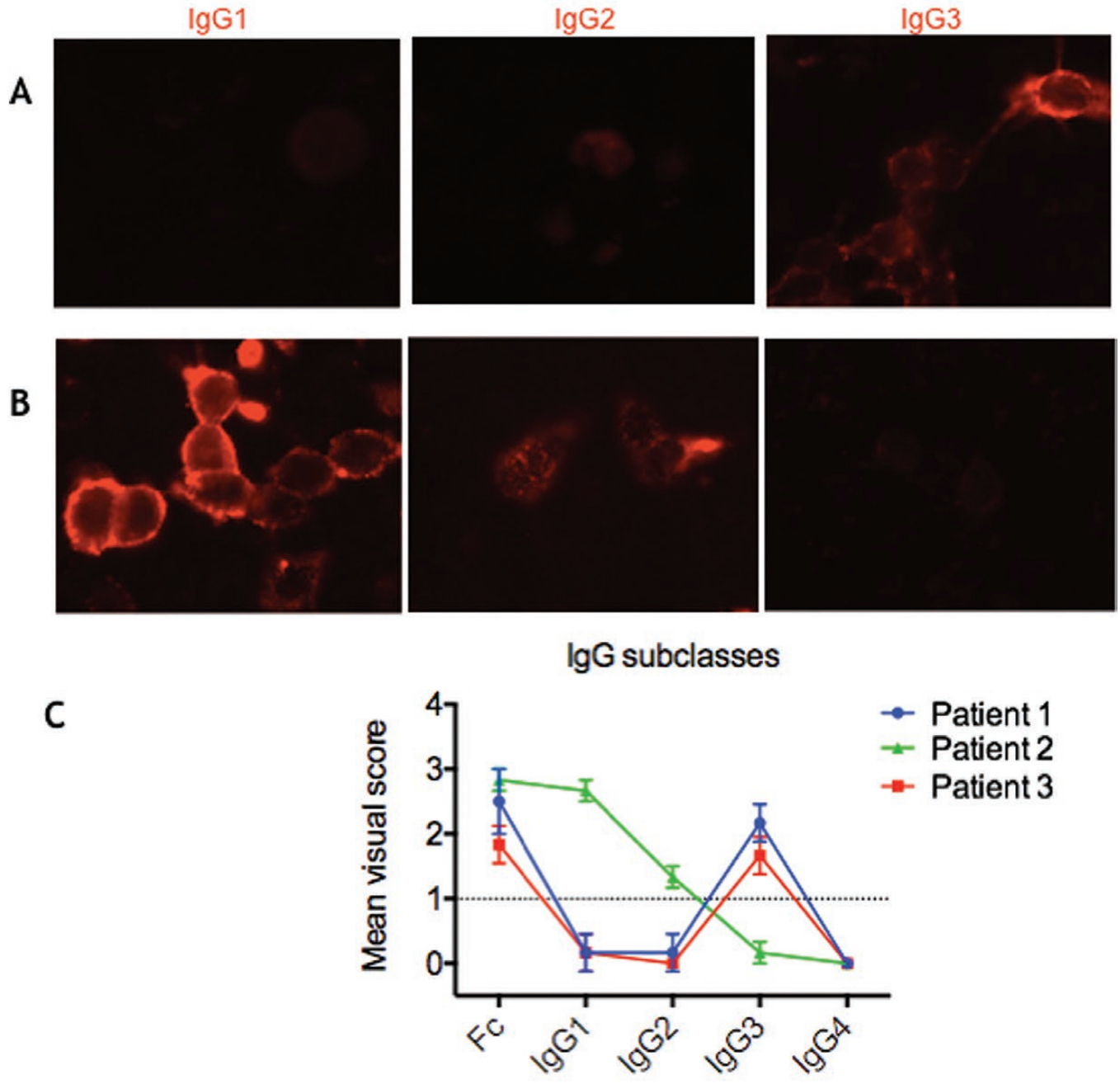

Figure 2—The three positive sera were retested for immunoglobulin G (IgG) using a IgG-specific anti-IgG Fc antibody and for the different IgG subclasses. Two patients had mainly IgG3 (A, C Patients #1 and 2) and one mainly IgG1 and IgG2 (B, C Patient #3). The results are given as mean ± standard error of mean (C).

disorder with severe psychotic features after the diagnosis of NT1. Patient #2 presented at age 16 with severe and progressively worsening EDS, daily nightmares, and rare episodes of sleep paralysis. From age 17, she developed episodes of muscle tone loss induced by emotions. Her MSLT (performed twice) was typical for the diagnosis of narcolepsy, but CSF hypocretin-1 levels were normal and the patient did not carry the HLA DQB*06:02. Although this is atypical, the clear-cut cataplexy, the typical MSLT and clinical history, led to a diagnosis of NT1. This was further supported by the good response of cataplexy to venlafaxine and subsequently to sodium oxybate treatment. Patient #3 presented with EDS since childhood; she never experienced cataplexy, and hypocretin CSF levels were normal, but her MSLT supported the diagnosis of NT2.

DISCUSSION

There has been much interest in looking for antibodies to potential neuronal targets in narcolepsy, and a recent study identified autoantibodies against the HCRTR2 in patients with post-Pandemrix® narcolepsy. We established a CBA to detect these antibodies, but only 3 (5%) narcolepsy patients had IgG antibodies to HCRTR2. Moreover, the titers were low in all 3, and no HCRTR2-Abs were found in their CSFs. Only one of three had classical NT1 and that patient also had psychosis. Tanaka et al.[17] also found a low proportion (5 of 181 patients, 4 NT1 and 1 NT2) of patients with antibodies immunoprecipitating [^{35}S]-HCRTR2. Thus, HCRTR2-Abs in patients with idiopathic narcolepsy appear to be rare. This, together with the low titers in serum and the absence of CSF antibodies, which are usually present in patients with typical antibody-mediated diseases,[23] suggest that they may not be clinically relevant.

There are, however, other possible reasons for the low titers and the absence in the CSF. None of the three patients with HCRTR2-Abs was assessed close to disease onset, a time at which the initiating immune response might be more evident. The absence in CSF may be related to the relatively low titers found in the serum where total IgG levels and most systemic antibodies are typically 300–400 times higher than those in the CSF.

Ours and Tanaka and colleagues'[17] results contrast with the much higher rate (85%) reported in patients post-Pandemrix® vaccination, although also in 34.7% controls.[21] Our results could reflect different methodologies, since we used a live-cell assay with visual inspection of the binding, which allows to exclude nonspecific staining, whereas the Pandemrix® study used a cell-based enzyme-linked immunosorbent assay[21] based on a colorimetric reaction. The different frequency may suggest a major role of these antibodies in vaccine-related narcolepsy but not in idiopathic cases or at least not in typical patients. Indeed, all our positives were atypical, as one presented NT1 and psychosis and two were DQB1*0602 negative, including one patient initially diagnosed with NT2 who subsequently developed cataplexy, despite normal HCRT-1 CSF levels. Rare cases of narcolepsy-like syndromes, often atypical, have been observed in the course of antibody-mediated disorders which in turn can be associated with low HCRT-1 levels, suggestive of a specific vulnerability of the hypocretin neurons independent of the HLA status.[26,27] Considering the high frequency of the DQB1*0602 allele in narcolepsy, the finding of antibodies in two HLA-negative patients supports a phenomenon occurring only in a rare subgroup of patients, which may be unrelated to classical narcolepsy.

Moreover, a major question regarding HCRTR2-Abs relates to whether they could be responsible for the loss of the HCRT-producing neurons in narcolepsy NT1 and where they might act in the brain. The pathogenic potential of autoantibodies is determined not only by their titers but also by their isotype. IgG1 and IgG3 are the most efficient in promoting C1q deposition and complement-dependent cytotoxicity. Therefore, if these antibodies are able to access the brain parenchyma, they could bind to and cause complement-mediated damage to HCRTR2-expressing cells. One would need to postulate, however, that the hypocretin neurons, which appear to be selectively destroyed in typical narcolepsy,[3,4] express autoreceptors making the cells vulnerable to the effects of the antibodies. This is controversial; Yamanaka and coworkers, using a neurophysiological/immunoelectron microscopic approach, found that orexin neurons are directly and indirectly activated by hypocretin via the HCRTR2.[28] By contrast, however, Vassalli et al. failed to demonstrate the expression of HCRTR2 on mouse hypocretin neurons.[22] Alternatively, since hypocretin neurons project widely in the brain where they modulate several crucial functions,[29] such as sleep and wake, feeding behavior and energy homeostasis, autonomic function, and reward system regulation, it is possible that the neuronal damage is not limited to the hypocretin neurons themselves but also affects other neurons that receive the hypocretin signals via HCRTR1 and HCRTR2. One postmortem narcolepsy study[30] suggested that gliosis was not restricted to the lateral hypothalamus which would be consistent with more widespread expression of an autoantigen. Until these issues have been resolved, the significance of any HCRT2-Abs, even in a subset of narcolepsy patients, needs to be interpreted with caution.

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Address correspondence to: Giuseppe Plazzi, MD, IRCCS Institute of the Neurological Sciences of Bologna, Ospedale Bellaria, Via Altura 3, 40139, Bologna, Italy. Telephone: +39 051 4966926; FAX: +39 051 4966176; Email: giuseppe.plazzi@unibo.it

CONFLICT OF INTEREST

MPG declares no conflict of interest. PW is supported by the NHS National Specialised Commissioning Group for Neuromyelitis optica, United Kingdom, and by the NIHR Oxford Biomedical Research Centre. He is a named inventor on patents for antibody assays and has received royalties. He has received speaker honoraria from Biogen Idec and Euroimmun AG, and travel grants from the Guthy-Jackson Charitable Foundation. FP declares no conflict of interest. RL declares no conflict of interest. GP has received consultant honoraria from UCB pharma, Jazz pharmaceuticals, and Bioproject. AV and the University of Oxford hold patents and receive royalties for antibody tests (MuSK, LGI1, CASPR2). No Clinical trial is indicated.